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Water Uptake by Dry Beans Observed by Micro-magnetic Resonance Imaging

KAORI KIKUCHI[1], MIKA KOIZUMI[2,*], NOBUAKI ISHIDA[2] and HIROMI KANO[3]

[1]*National Institute of Vegetable and Tea Science, Ano, Mie 514-2392, Japan,* [2]*National Food Research Institute, Tsukuba, Ibaraki 305-8642, Japan and* [3]*Oak-Hill Georgic Patch-Work Laboratory, 4-13-10 Miyamoto, Funabashi, Chiba 273-0003, Japan*

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• *Background and Aims* Water uptake by dry kidney beans (*Phaseolus vulgaris* 'Rajma') and adzuki beans (*Vigna angularis*) was traced using micro-magnetic resonance imaging in order to elucidate the channel of water entry, the manner of water delivery and the timing of swelling of the seeds.
• *Methods* Magnetic resonance images of beans absorbing water were continuously measured with the single-point imaging method for 16 h or 20 h at 15-min intervals. With this technique, it was possible to detect and visualize the location of water in the beans, at a low water content, in the initial stages of water entry.
• *Key Results* Water was taken up through a specified tissue, the lens, near the hilum, and distributed primarily to the testa. When water reached the radicle, it began to be incorporated into cotyledons with considerable swelling of the seeds. Water uptake took place within a short time for kidney beans. The initial process of water entry was associated with mechanical vibration of the seed. Rapid hydration of the testa and the swelling of the cotyledons were then observed. Water was supplied to cotyledons through the adaxial epidermis. In contrast, it took a long time, approx. 7 h, to activate the water channel of the lens for adzuki beans which have a tightly fitting testa. Steeping of the testa was not uniform, which induced temporary slanting before enlargement of the seed.
• *Conclusions* The activation of the lens as the sole water channel, the delivery of water to the radicle within the testa, the swelling of the cotyledons, and the further increment of water are physiologically different processes during imbibition, and were separated by locating water in various tissues and by analysing the time course of water uptake using magnetic resonance imaging with the single-point imaging method.

Key words: Water uptake, dry beans, the role of testa, lens, real-time imaging, MRI, *Phaseolus vulgaris*, *Vigna angularis*.

INTRODUCTION

Seeds undergo desiccation during maturation and ripening and so biological reactions cease. This is a natural feature of seeds that enables them to endure severe conditions such as cold temperature while maintaining viability before germinating by absorbing water in optimal conditions. The amount of water dry seeds take up on imbibition is large, and greatly increases seed size prior to the initiation of active metabolism. The hydration of bio-membranes is necessary for initiating biological actions, and the hydration of stored starches and proteins is needed to supply substrates for active metabolism, producing energy and the materials required for seedling growth. Various materials located in different tissues in the seeds exhibit individual properties of hydration, different water absorption rates and size increments. However, these are biologically regulated, in complicated processes, to prevent a fatal imbalance between the tissues as an organic whole. Therefore, tracing the hydration mechanism of dry seeds in relation to morphology at early, near-dry stages of imbibition is expected to provide a better understanding of seed germination and of the preparation of seeds as foods.

Dyes have been used to investigate water status in plants—classically safranine for water distribution in tissues, and iodine (Collins, 1918) and gentian violet (Dell, 1980) for water movement in seeds. Water uptake by dry seeds has been traced by measuring the increase of weight against initial dry weight, by the changes in morphology under a microscope or an electron microscope (Webster and Leopold, 1977; Dell, 1980; Manning and Van Staden, 1987; McDonald *et al*., 1988*a*, *b*), X-ray analysis using a scanning electron microscope (SEM) (Davies, 1991), X-ray photography (Liu *et al*., 1993) and by tritiated water as a tracer (Jenner, 1985). The neutron beam-CT method, which is a new technique for investigating water in plants, located water in seeds with a high spatial resolution and with a time resolution of 1 h during imbibition (Nakanishi and Matsubayashi, 1997). The X-ray CT method used to evaluate quality of vegetables and fruits (Lammertyn *et al*., 2003) and for detecting water in single roots (Hainsworth and Aylmore, 1986) is considered to be a useful technique in the study of seed germination. Another way is to use nuclear magnetic resonance (NMR) imaging or magnetic resonance imaging (MRI), which is useful for tracing the dynamic movement of water in plant tissues (Callaghan, 1991) and for studying the biological implications in relation to water

* For correspondence. E-mail mkoizumi@affrc.go.jp

distribution (Yamazaki *et al*., 1995; Ishida *et al*., 2000), including seed germination (Pietrzak *et al*., 2002) and evaluation of seed quality (McEntyre *et al*., 1998). Near-real-time measurements of water movement in plant tissues were demonstrated by using a paramagnetic enhancer or tracer (Connelly *et al*., 1987; Koizumi *et al*., 1992) and phase-encoding methods (Callaghan, 1991; Köckenberger *et al*., 1997; Rokitta *et al*., 1999).

Water uptake by dry seeds has been studied using MRI for maize (Ruan and Litchfield, 1992; Ruan *et al*., 1992), barley (Gruwel *et al*., 2002; Molina-Cano *et al*., 2002), wheat (Song *et al*., 1998), legumes (Heil *et al*., 1992; Pietrzak *et al*., 2002), tobacco (Manz *et al*., 2005) and western white pine (Terskikh *et al*., 2005). However, this technique has not been effectively used for the earliest stages of leguminous seed imbibition associated with hydration and activation of the water channel at water contents ranging from 10 % to 30 %. General imaging methods, e.g. the spin-echo method, cannot efficiently detect the presence of water in very low amounts, the so-called bound water, from echo-formation with the shortest time of 1 ms, even using the elegant fast low-angle shot (FLASH) method (Ruan and Litchfield, 1992). Signals from water of very low mobility relaxing in <1 ms are excluded. Recent advances in single-point mapping imaging (SPI) of MRI (Cho *et al*., 1995) provided the means to locate water in materials with such low water concentrations, using a short T_2 propagation, a dephasing time of one-tenth of the echo formation, and a short repetition time. In the current investigation, therefore, the mechanism of water uptake by dry beans was studied by real-time imaging with a high time resolution. This investigation clarified where water enters the seed, how water is delivered to individual tissues and the processes involved during imbibition by two bean species.

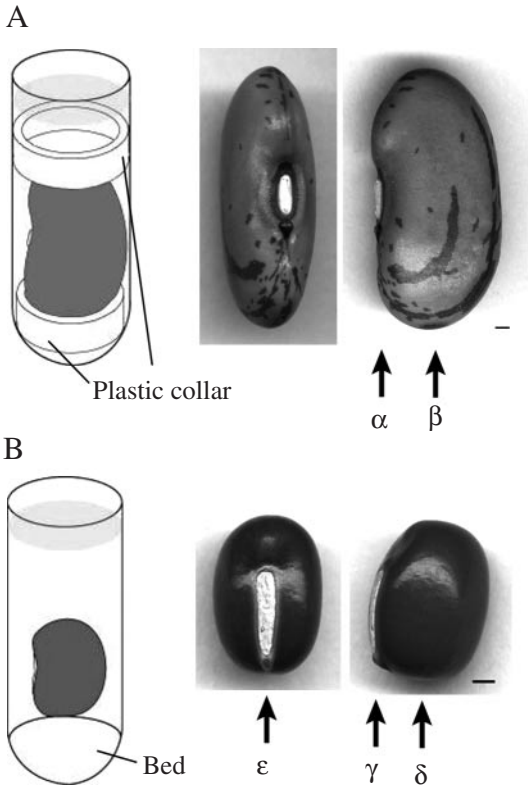

FIG. 1. A kidney bean (*Phaseolus vulgaris*) 'Rajma' and an adzuki bean (*Vigna angularis*) set in sample tubes for MR image measurements. (A) A kidney bean was fastened by two inner plastic collars near the top and bottom of the sample tube. Sliced sections of images are at β in Fig. 2 and from β = 69 to α = 87 in Fig. 3. (B) An adzuki bean was fixed at the bottom of the long axis by plastic binder on a small bed in the sample tube. Sliced sections of images are at ε in Fig. 5, at γ in Fig. 6 and at δ in Fig. 7. Image sections are observed from 90° angle directions. Scale bars = 1 mm.

MATERIALS AND METHODS

Plant material

Kidney beans (*Phaseolus vulgaris* 'Rajma') and adzuki beans (*Vigna angularis*) were used. Water contents of stored beans were 12·0 ± 0·02 % for kidney beans and 14·7 ± 0·05 % for adzuki beans, and they increased up to 35·8 ± 0·09 % for the former and 33·7 ± 0·30 % for the latter during imbibition. A kidney bean was set in a 15-mm sample tube and positioned vertically between plastic collars (Fig. 1A); an adzuki bean was fixed with a plastic binder on a small bed at the bottom of a 12-mm sample tube (Fig. 1B). The sample tube was then filled with water and inserted into an MRI probe, and images were acquired continuously.

Measurements of MR images

An NMR spectrometer (DRX 300, Bruker, Karlsruhe, Germany) and an imaging accessory were used for measurements. Images were measured by the SPI method using the protocol for the system. Four replicate measurements were made for kidney beans and two for adzuki bean; one of each is presented.

Three-dimensional measurements were carried out. Kidney beans were measured at 15-min intervals for 16 h from 5 min after imbibition of water. The field of view (FOV) was 20 × 20 × 30 mm with a 64 × 32 × 32 matrix. Adzuki beans were measured at 15-min intervals for 20 h from 10 min after imbibition of water. The FOV was 15 × 15 × 15 mm with a 64 × 32 × 32 matrix. Dephase time was set at 0·1 ms, and repetition time (T_R), 5 ms. It took approx. 5 min per image.

Measured data sets with single accumulation were Fourier transformed, transferred to a micro-computer, converted to data format and zero-filled, and images were created in a 128 × 128 × 128 matrix. Resulting spatial resolutions were 156 × 156 × 234 μm for kidney beans and 117 × 117 × 117 μm for adzuki beans. The digitally sliced sections of images in the individual figures are shown in Fig. 1 (arrows α–ε). Image data were further processed with the Scion Image program [the public domain NIH Image Program (National Institutes of Health, USA) translated for Windows operating systems by Scion Co., Frederick, MD, USA; available on the Internet at http://www.scioncorp.com] and locally developed programs in Visual BASIC (Microsoft Co., Tokyo, Japan), which were then displayed with the Photoshop program

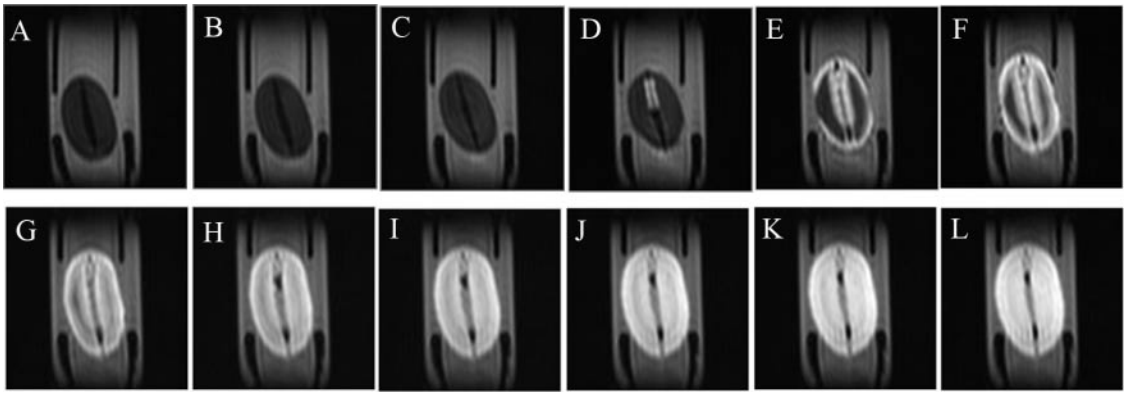

FIG. 2. Changes in a kidney bean during imbibition at a median longitudinal section (Fig. 1, β) normal to the raphe–antiraphe of the longer axis. Images were acquired continuously for 16 h with 15-min intervals after 5 min of imbibition. The images acquired at 60-min intervals are shown from 20 min of imbibition. (A) 20 min; (B) 80 min; (C) 140 min; (D) 200 min; (E) 260 min; (F) 320 min; (G) 380 min; (H) 440 min; (I) 500 min; (J) 560 min; (K) 620 min; (L) 680 min. Highlighted signals represent free water taken up.

(Adobe Systems Inc., Tokyo, Japan) and rendering software, NMRv2b or NMRview (MR Technology, Tsukuba, Japan). Typical results are shown with appropriate time separations. The processes involved with imbibition were quantitatively analysed using the Scion Image program and Excel program (Microsoft Co., Tokyo, Japan).

RESULTS

Incorporation of water into a kidney bean from lens to radicle through testa prior to the entry into cotyledons

The image changes during imbibition of a kidney bean at a median longitudinal section normal to raphe–antiraphe of the seed (Fig. 1A, β section) were acquired with a 15-min interval for 16 h; the image at 20 min and images at intervals of 60 min are shown in Fig. 2. Water was not detected in the slice plane for approx. 3 h (Fig. 2A–C). Strong water signals first appeared in the adaxial epidermis of cotyledons (Fig. 2D) and subsequently in the testa, including the abaxial epidermis of cotyledons (Fig. 2E). Obvious swelling of the seed was observed after 4 h of imbibition. Water signals circled around cotyledons, and then water started to spread into internal areas of cotyledons from adaxial tissues at 5 h (Fig. 2F). Rapid elongation of cotyledons was terminated at 8 h (Fig. 2I), but signal intensity subsequently increased without notable increment of the seed.

Sorting sliced sections from position β (slice plane 69) to position α (slice plane 87) in Fig. 1 revealed different features of the event (Fig. 3). Water entered through the lens under the hilum (section 87) at 95 min of imbibition (Fig. 3A) and spread upwards around the hilum in a circular shape (Fig. 3A and B). Considering that a stronger signal was located in the seed coat or testa on the images at 170 min (section 81; Fig. 3H) and 200 min (sections 81 and 77; Fig. 3 M and N), water first entered the testa before entering cotyledons. Upon reaching the radicle through the testa at 200 min (section 85; Fig. 3L), water might have begun to enter the adaxial epidermis of cotyledons (sections 77 and 69; Fig. 3N and O).

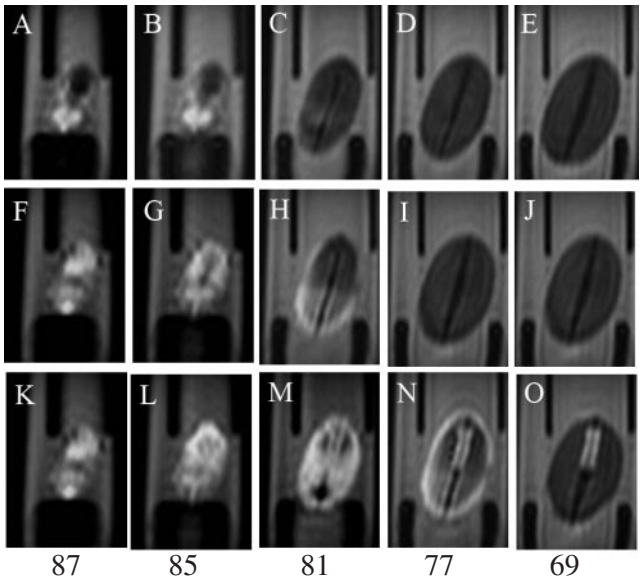

FIG. 3. Water distribution inside of the kidney bean at 95 min (A–E), 170 min (F–J) and 200 min (K–O). Longitudinal sections are shown. Figures under the images indicate sliced sections from the central position (69) to the edge of the bean with lens (87). Sections 69 and 87 correspond to β and α in Fig. 1, respectively.

A movie **[Supplementary Information—Video]** made of the continuously acquired images revealed that the bean was subjected to vibration at 65 min after activation of the lens. The vibration of the seed indicates that a certain imbalance of forces was produced by water being incorporated inside the dry seed.

Incorporation of water into an adzuki bean and the initial process of water entry from the lens

A similar experiment was carried out using an adzuki bean, which has a tight testa that makes it difficult to dry in contrast to the kidney bean that has a rough testa that cannot be preserved for a long time. Figure 4 shows the morphological images of the adzuki seed acquired by a

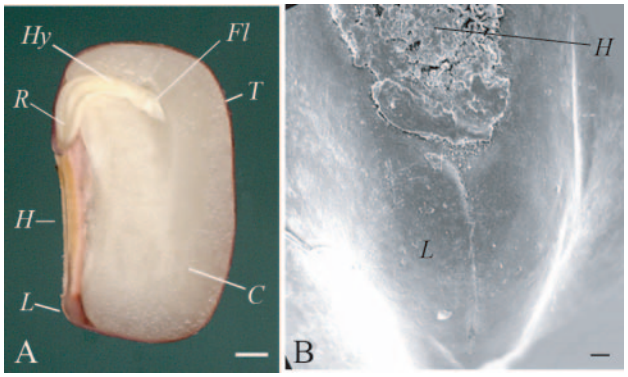

FIG. 4. Pictures acquired by an optical (A) and a scanning electron microscope (B) showing the anatomy of adzuki beans. (A) The seed coat or testa (*T*) includes cuticle, epidermis, sclereid layer, parenchyma, remnant layer and retarded endosperm. Cotyledons (*C*) are present beneath them. The lens (*L*) is at the opposite side of the radicle (*R*), hypocotyl (*Hy*) and first leaf (*Fl*). The hilum (*H*) is between the lens (*L*) and the radicle (*R*). (B) The lens (*L*) exhibits a rough surface. Scale bars: A = 1 mm; B = 100 μm.

low magnification optical microscope and of the lens tissues acquired by SEM. In the area of the lens, there were raised areas on the surface of the testa in the SEM image (Fig. 4B). Morphological characteristics concerning the hilum, the lens under the hilum, the radicle, the seed coat or the testa and cotyledons (Fig. 4A) were referenced to the anatomical interpretation of leguminous seeds by Gunn (1981) and Van Staden *et al*. (1989).

Figure 5 presents the changes of a median longitudinal section through the raphe–antiraphe near adaxial epidermis of a cotyledon (Fig. 1, ε) with a 2-h separation from 1 h to 19 h of imbibition. Water was detected beneath the hilum where the lens might be, after 3 h soaking in water (Fig. 5B), and stayed for >5 h (Fig. 5C). Water subsequently spread into the inner tissues after 7 h (Fig. 5D) through vascular-like tissues, probably in the seed coat, that seemed inactive (Fig. 5E) because water immediately filled parenchyma cells with similar intensity (Fig. 5F). The seed slanted extremely to the right from 11 h to 13 h with intensification of signals in the testa and widening of the signal area in the cotyledon (Fig. 5F and G). When the signal covered 80 % of the seed (associated with the termination of slanting at 13 h), the size rapidly doubled. The seed elongated and widened, and the signal intensity increased (Fig. 5H–J).

Figure 6 illustrates the changes in the transverse section of the longer axis near the hilum (Fig. 1, γ). The signal was detected in the hilum 1 h after soaking (Fig. 6A) and appeared in the lens at 3 h (Fig. 6B). The signal began to spread circularly at both sides of the hilum after 7 h (Fig. 6D and E). The bean elongated and widened after 13 h (Fig. 6G and H). The signal intensified from 17 h to 19 h without an increase in seed size (Fig. 6I and J).

Figure 7 depicts the process of water delivery in the central transverse section (Fig. 1, δ) of the seed. Stronger signals were observed in the testa, and, at the same time, weaker signals were detected in the internal areas of the cotyledons with slight swelling (Fig. 7A–C). After water reached the radicle, which swelled resulting in a protrusion at the top of the seed at 13 h (Fig. 7D), the seed exhibited extreme elongation and swelling, and became ellipsoidal (Fig. 7E). The signal in the testa was strong, and that in the cotyledons intensified slightly (Fig. 7F).

Water entry in the seed was limited to the lens near the hilum; water did not permeate other parts of the seed coat or testa. The presence of water signal at the lens for a long period may involve hydration of the lens membrane to allow recovery of physiological function, a process that takes a rather long time for adzuki beans. The adzuki bean apparently tilted on its axis between 11 h and 13 h (Fig. 5F and G), which may imply a phenomenon similar to the vibration of the kidney bean. It is interesting that cotyledons are not considered to swell rapidly until the radicle is hydrated (Figs 5–7). Water primarily spread first in the testa from the lens to the radicle, then into inner tissues of cotyledons to induce swelling and, at the same time, filled the remaining tissue of the testa. These features were similar to those of the kidney bean.

Time course of water uptake and the increase in seed size

The amount of water taken up, the increases in seed size (area), and the elongation of the longer and the shorter axes for the kidney bean 'Rajma' were calculated using the original series of images in Fig. 2 and shown in Fig. 8, and those for the adzuki bean, using the images in Fig. 5 and shown in Fig. 9. The former exhibited a short lag time before the entry of water and rapid seed swelling; the latter, a long lag time before water flowed into the seed and complicated increments in seed size. During the lag time, the lens was hydrated for 65 min to 95 min for kidney beans (Fig. 3A) and 3 h to 7 h for adzuki beans (Figs 5B–D and 6B–D). The radicle protruded at approx. 200 min for kidney beans (Fig. 3L) and 13 h for adzuki beans (Fig. 7D), after which the increments in seed size and the rapid uptake of water took place. The increments in seed size terminated before the intensification of water signals stopped. During measurements, water content increased from approx. 12 % to 36 % in kidney beans and 15 % to 34 % in adzuki beans.

DISCUSSION

The early stages of water uptake by dry beans when the water content was only 10–35 % were studied. The results demonstrated that water entered through the lens, as the sole natural water channel (Figs 3, 5 and 6), and reached the radicle prior to the cotyledons swelling (Figs 3 and 7). These processes took place without a remarkable increase in seed water content (Figs 8 and 9). The two seeds, kidney bean and adzuki bean, seemed to emphasize different aspects in a similar process of water uptake according to the characteristics of each seed, or the properties of the seed coat or testa (Duke and Kakefuda, 1981; McDonald *et al*., 1988*a*; Van Staden *et al*., 1989).

For the western white pine (Terskikh *et al*., 2005), the phase of imbibition was successfully separated on the MR

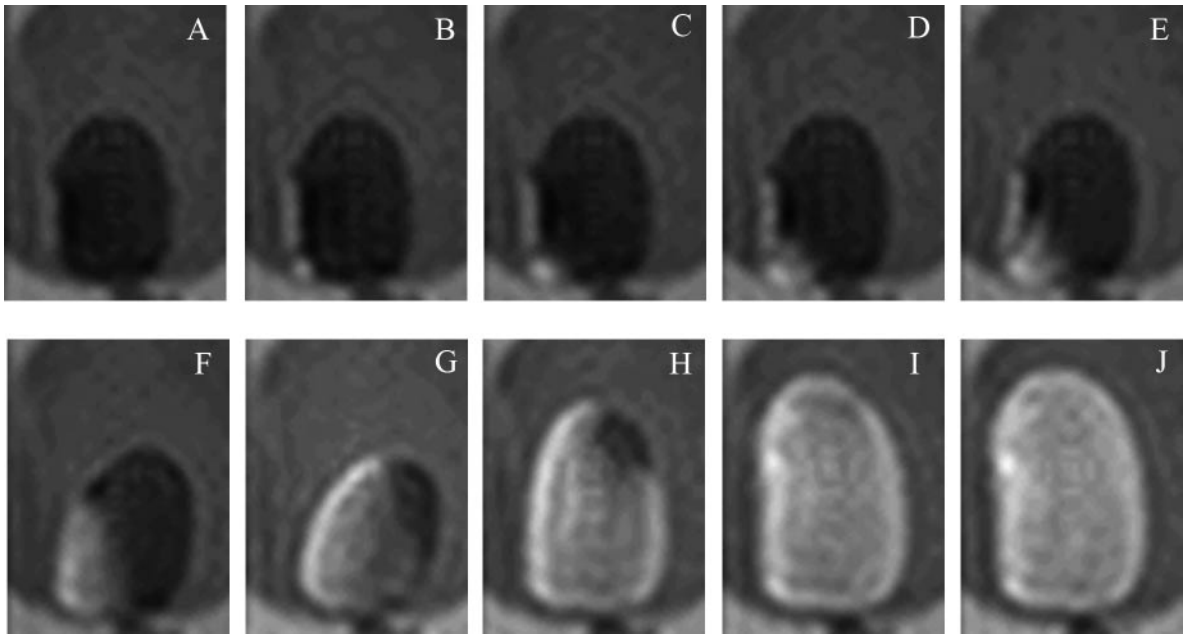

FIG. 5. Changes in an adzuki bean during imbibition at the longitudinal section parallel to the cotyledons (Fig. 1, ε) of the longer axis near the adaxial epidermis. Images were continuously acquired for 20 h from 10 min after imbibition with 15-min intervals. Images acquired at 2-h intervals from 1 h of imbibition are shown. Water was detected at the lens under the hilum at 3 h (B). Water seemed to be delivered through vasculatures and, at the same time, filled parenchyma cells at 9 h (E). (A) 1 h; (B) 3 h; (C) 5 h; (D) 7 h; (E) 9 h; (F) 11 h; (G) 13 h; (H) 15 h; (I) 17 h; (J) 19 h.

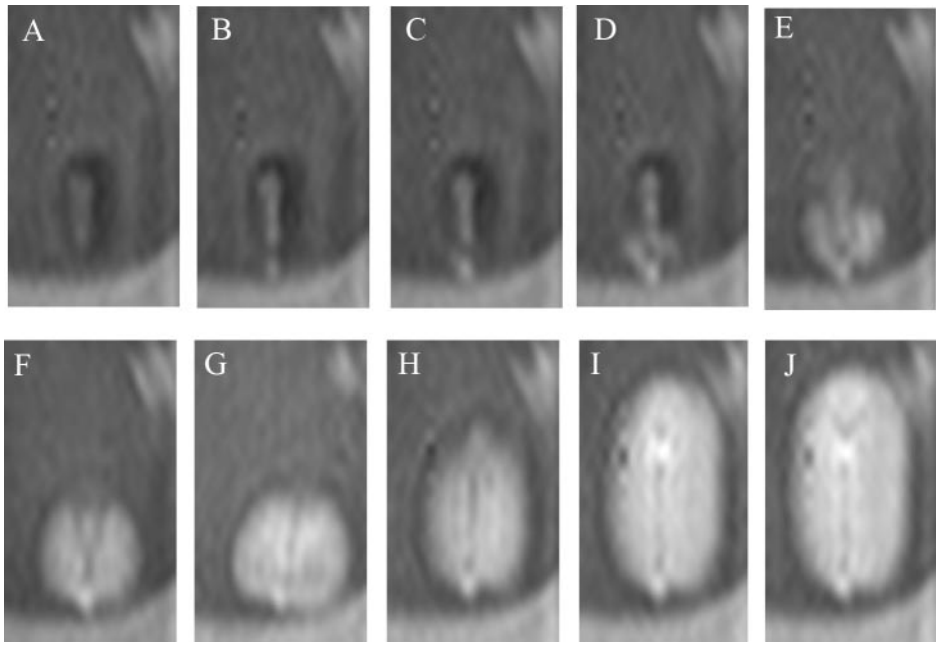

FIG. 6. Changes in an adzuki bean during imbibition at the longitudinal section normal to the raphe-antiraphe (Fig. 1, γ) near the hilum for 19 h. Water was detected at the lens under the hilum at 3 h (B) and began to spread from the lens with a spherical shape on both sides of the hilum at 9 h (E). The round area where water spread increased towards the radicle at 11 h (F). (A) 1 h; (B) 3 h; (C) 5 h; (D) 7 h; (E) 9 h; (F) 11 h; (G) 13 h; (H) 15 h; (I) 17 h; (J) 19 h.

images into three stages: (I) water penetration into testa and accumulation between the seed coat and megagametophyte; (II) hydration of the cotyledons; (III) water migration to the hypocotyl followed by later spreading towards the radicle. Similar phases in water uptake are stated for tobacco seed (Manz *et al*., 2005), but the second phase for tobacco seeds is considered to contain both the second and third phases of the western white pine (Terskikh *et al*., 2005), based on the increasing curves of water content in the former and the signal intensity in the latter. The measurements for cereal crops, maize (Ruan and Litchfield, 1992; Ruan *et al*., 1992), barley (Gruwel

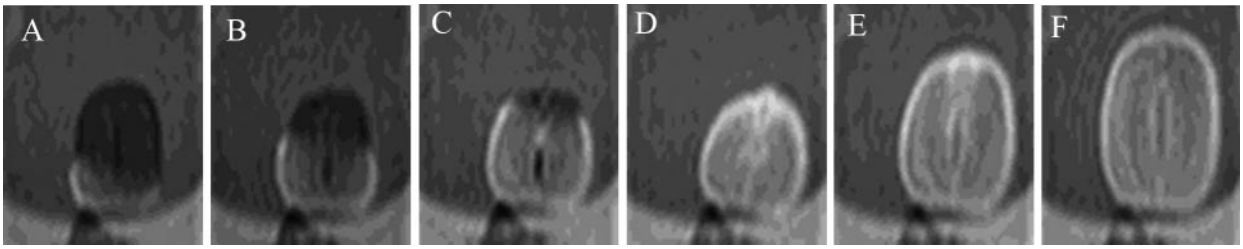

FIG. 7. Water distribution in an adzuki bean at the median longitudinal section (Fig. 1, δ) from 9 h 25 min to 15 h 40 min. The images are similar to those of the kidney bean in Fig. 2. Water was primarily located in the testa, and, at the same time, a small amount of water was detected in cotyledons before water reached the radicle. (A) 9 h 25 min; (B) 10 h 40 min; (C) 11 h 55 min; (D) 13 h 10 min; (E) 14 h 25 min; (F) 15 h 40 min.

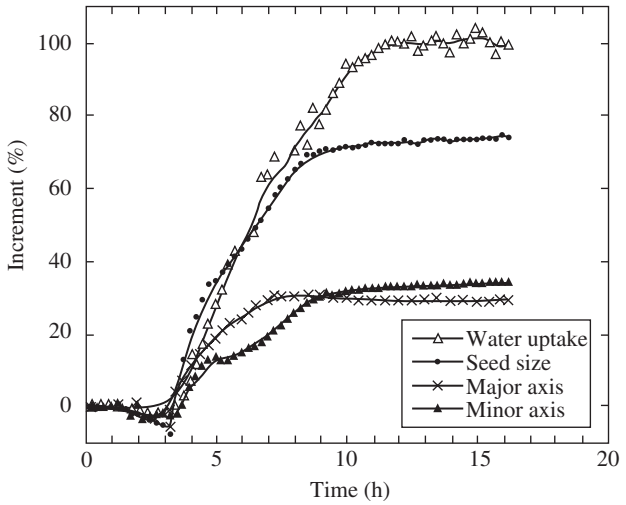

FIG. 8. Time course of water uptake, the increase of size, and the increment of major axis and minor axis of the kidney bean. Measurements were carried out on the same sections as in Fig. 2. Water amount (density) was indicated by the integrated signal intensity: the increase of size, by the area of the bean. The increments (%) in the vertical axis were indicated by $(V_t - V_0)/V_0 \times 100$, where V_t is the value at time t and V_0 is the value at time 0.

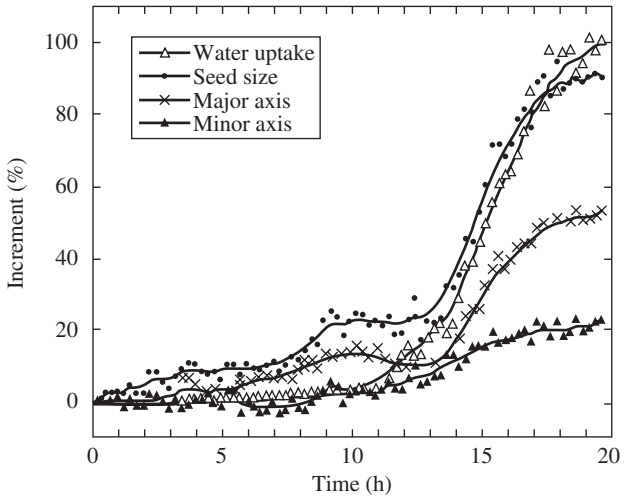

FIG. 9. Time course of water uptake, the increase of size, and the increment of major axis and minor axis of the adzuki bean. Measurements were carried out on the same sections as in Fig. 5. Water amount (density) was indicated by integrated signal intensity: the increase of size, by the area of the bean. The increments (%) in the vertical axis were indicated by $(V_t - V_0)/V_0 \times 100$, where V_t is the value at time t and V_0 is the value at time 0.

et al., 2002; Molina-Cano *et al*., 2002) and wheat (Song *et al*., 1998), seem to be the third phase because these seeds have no developed cotyledons and water reaches the embryo fast. In this context, the current investigation corresponds to the first phase, including the early half of the second phase of the western white pine, when water is rapidly incorporated into the testa and cotyledons, and water content ranges from 10 % to approx. 40 % in leguminous seeds.

Detection of a low water content with a short imaging time was necessary to obtain time-lapse traces of the initial water incorporation into seeds. Ruan *et al*. (1992) used the celebrated FLASH method with a 1-ms echo-time for steeping maize seeds with the shortest imaging time of approx. 15 min, which clearly recorded wetting the embryo with a higher water content of 50 %. A water content of 13 % to 17 % restores the functional structure of membrane systems with selective permeability in leguminous seeds, which possess high binding energy (Vertucci and Leopold, 1984), and is severely restricted in motion. This may result in a signal decline before the echo-formation of the general methods due to the very short spin–spin relaxation. Therefore, bound water could not be probed until the signal could be acquired within 100 or 200 μs after excitation of spins by the SPI method. The SPI method (Cho *et al*., 1995) acquires a strong signal from low-mobility bound water. Moisture loss from rice seeds as low as 20 % to approx. 6 % was quantitatively analysed by the SPI method with a time separation between successive images of 10 min (Ishida *et al*., 2004). This was especially useful for locating the first hydration point on the seed coat, the lens, and following the path of water in the seed coat when the seed water content did not obviously increase. In addition, the signal from steeping water outside beans was effectively avoided to discriminate the events occurring inside the seed by using an extraordinarily short repetition time.

Water absorption is reported to occur from the lens, based on a germination test with a sealing lens of *Sesbania punicea* (Manning and Van Staden, 1987). Similarly, in the current investigation, the lens was visualized to be the sole channel for water to enter the seed (Figs 3A, 5B–D and 6B–D), although preceding studies using SEM and MRI emphasize the importance of the hilum/micropyle region (Heil *et al*., 1992; McDonald *et al*., 1988*a*; Pietrzak *et al*., 2002). The lens is a specified tissue with a rough

surface near the hilum on the opposite side of the micropyle (Fig. 4B). By lifting or ejecting the strophiolar plug (Dell, 1980; Van Staden *et al*., 1989), the lens cavity of *Albizia* seed governs the rate of water incorporation into seeds afterwards. For adzuki beans, it took a long time to initiate water entry; the water signal stayed at the lens for >5 h (Fig. 5). Slow and gentle hydration was needed to restore the membrane function of lens cells, a possible mechanism for which was proposed by Simon (1974) for dry bio-membranes. The nature of lens tissues, together with a hard testa, may provide adzuki beans with high tolerance against drying (Fig. 4) (Van Staden *et al*., 1989). In contrast, kidney beans activate the lens quickly (Fig. 3), and are known to be sensitive to dry conditions and to suffer soaking damage during long-term storage (Pollock *et al*., 1969).

The patterns of the changes in seed size throughout the activation of lens tissue and water penetration into the seed before reaching the radicle differed between the kidney bean (Fig. 8, before 3 h) and the adzuki bean (Fig. 9, before 13 h). The former exhibited a slight decrease in size (Fig. 8, around 3 h) as reported for soybean (Parrish and Leopold, 1977), while the latter exhibited a rapid but small increase in size on the activation of the lens at 8 h followed by a slight decrease in size (Fig. 9, from 9 to 13 h). The discrepancy may result from the combination of the expansion effect with the steeping testa, and the negative effect due to the phase transition in internal gases accompanying wetting because the solubility of carbon dioxide is much greater than that of oxygen. The increment in size of the adzuki bean was greater than that of the kidney bean. During the period of increase, water fronts progressed in parallel from the lens to the radicle in both testa and cotyledons (Figs 3H, 5E–G and 7A–C), but water was primarily delivered in the testa, as indicated by intensified signals. Accordingly, the processes can be ascribed to the wetting of the testa, which made the testa elastic. Water filled the epidermis, parenchyma and endosperm, possibly through vasculature (Manning and Van Staden, 1987) as reported for *Albizia lophantha* (Dell, 1980). At the same time, water diffused to cotyledons, but the amount was very small judging from the weaker signal intensity compared with that in the testa. The membranes of cytoplasm, mitochondria and vesicles that first encounter limited water must be hydrated to restore physiologically functional structures (Simon, 1974; Webster and Leopold, 1977). Hydrated vacuolar membranes prevent further incorporation of water in order to avoid the destruction of cell membranes by sudden swelling of the stored materials. These processes of water uptake are non-energetic. Hydration of testa and endosperm is not directly related to germination but plays a role as a buffer to water stress and a regulator of water balance in germination for *Trigonella foenum-graecum* seed (Reid and Bewley, 1979).

Water migrated through the testa and reached the radicle, and the radicle swelled into a protrusion (Figs 3L and 7D). This resembles the case of the broad bean measured by the neutron beam-CT method. Water increases outside a broad bean seed, and the intensity in the radicle increases and testa enlarge at 2 h of imbibition (Nakanishi and Matsubayashi, 1997). A larger increase of the embryonic axis was reported for soybean (McDonald *et al*., 1988*b*). This may initiate biological actions of the seeds. Water passed through the adaxial surface and was delivered to internal areas of the cotyledons through the radicle and hypocotyl. Considerable, fast swelling then took place, and signals in cotyledons then intensified (Figs 2 and 5). Seed size increased from 175 % to 190 % of the initial sizes (Figs 8 and 9). The large degree of swelling of stored macromolecular compounds has to occur at an appropriate rate (Figs 2 and 5–7) in order to prevent damage to internal seed tissues. Water uptake into cotyledons (Fig. 8, after 3 h, and Fig. 9, after 13 h) should thus be controlled biologically. Water increase in the cotyledons has essentially different implications from the former hydration of the testa, because water acts as the medium for the degradation metabolism of stored materials that are regulated by the embryonic axis (Van Staden *et al*., 1989).

The signal intensity did not stop rising when the seed stopped expanding; water incorporation exceeded the expansion of the seed. This may be because inter- and intra-gas spaces of cells were diminished. Driving gases from small pores in cell constituents by replacement with water requires a large amount of free energy, a biological event. Another possibility is that the molecular structures of stored materials were rearranged to hold more water between the molecular structures, such as the transformation of starches from crystal to gel, and from gel to solution with release of cations. The conformational changes, or unfolding and refolding of macromolecular compounds, are biological processes. Stored starches change their form from crystal to amorphous with hydration; hydrophilic proteins [late embryogenesis abundant (LEA) proteins] abound (Colmenero-Flores *et al*., 1999) and are loosely structured to capture water and to provide protection in low water potential; and functioning proteins, oleosin, associate with stored oils and control oil metabolism during germination (Froese *et al*., 2003) in seeds. In this context, the last phase of signal intensity increasing after the seed has stopped growing (Fig. 8, after 8 h and Fig. 9, after 18 h) should be recognized as a different physiological stage of water absorption from the former stages of seed enlargement.

Water incorporated from a specified point, the lens, hydrated cell constituents non-uniformly, which generated a local imbalance of expansion force, causing the adzuki bean to incline and revolve around a fixed point from 11 h to 13 h of imbibition (Fig. 5F and G). A corresponding oscillation of the seed was observed in kidney beans from 65 to 125 min of water uptake. These were observed after the activation of the lens and just before active enlargement of the seeds. The time course of water uptake indicates that imbibition by dry seeds is, to some extent, accompanied by uneven swelling during the first entry of water. Water enters seeds with cell membranes of non-recovered functions and produces an imbalance of forces between local tissues from the swelling of cell constituents. Hydration occurred slowly in the adzuki bean;

therefore, the adzuki bean is resistant to drying. Fast flow of water causes the kidney bean to vibrate. If water enters too rapidly without the control of the lens in beans with cracked testas, the non-uniform swelling will damage functional tissues, i.e. it will cause soaking effects (Hobbs and Obendorf, 1972; Duke and Kakefuda, 1981; McDonald *et al*., 1988*a*).

The SPI methods enabled continuous imaging of seed imbibition with a shorter time resolution than ever reported before. Water incorporated from the lens was first, transported to the radicle through the testa, which may be a non-biological step. Upon reaching the radicle, water began to enter the cotyledons, which induced a major increase in seed size; this may be under biological control. The two stages during imbibition before and after the radicle was hydrated, were clearly distinguished by continuous imaging. This is a new finding. Water uptake continued even after the seeds stopped increasing in size, which may be the consequence of water filling gas spaces, and the conformational changes of macromolecular compounds to hold more water. These might be accompanied by physiological reactions in cells. This piece of work visualizing the hypothetical path of water entry, constructed using various methods, makes a convincing contribution to a better understanding of imbibition events in leguminous seeds.

SUPPLEMENTARY INFORMATION

A QuickTime movie of the continuously acquired images of kidney bean 'Rajma' during imbibition is available online at http://aob.oxfordjournals.org/. Water enters the cotyledons from the adaxial surface then the abaxial surface, and extends inside the cotyledons primarily from the adaxial side.

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